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Eu(III) luminescence and tryptophan fluorescence spectroscopy as a tool for understanding regioselective hydrolysis of hen egg white lysozyme by metal-substituted Keggin type polyoxometalates

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Abstract

The interaction between the lacunary Keggin $K_7PW_{11}O_{39}$, the Eu(III)-substituted Keggin $K_4EuPW_{11}O_{39}$ (Eu-Keggin) and the Ce(IV)-substituted Keggin $[Me_2NH_2]_{10}[Ce(PW_{11}O_{39})_2]$ (Ce-Keggin) polyoxometalates (POMs), and the proteins hen egg white lysozyme (HEWL) and the structurally homologous α-lactalbumin (α-LA) was studied by steady state and time-resolved Eu(III) luminescence and tryptophan (Trp) fluorescence spectroscopy. The excitation spectrum of Eu-Keggin at lower concentrations ([Eu-Keggin] <100 μM) is dominated by a ligand-to-metal charge transfer band (291 nm). For higher concentrations ([Eu-Keggin] >250 μM) the $^5L_6 \leftarrow ^7F_0$ transition becomes the most intense peak. In the absence of protein, the number of coordinated water molecules to the Eu(III) center of Eu-Keggin is 4, indicating a 1:1 Eu(III):POM species. In the presence of phosphate buffer this number linearly decreases from 4 to 2 upon increasing phosphate buffer concentration. Upon addition of HEWL, there are no coordinated water molecules, suggesting interaction between Eu-Keggin and the protein surface. In addition, this interaction results in a more than threefold increase of the hypersensitive $^5D_0 \rightarrow ^7F_2$ transition for the Eu-Keggin/HEWL mixture. The calculated association constant amounted to 2.2×10^2 M^{-1} for the Eu-Keggin/HEWL complex. Tryptophan fluorescence quenching studies were performed and the quenching constants were calculated to be 9.1×10^4 M^{-1}, 4×10^4 M^{-1} and 4.1×10^5 $M^{-1}$for the lacunary Keggin/HEWL, the Eu-Keggin/HEWL and the Ce-Keggin/HEWL complexes, respectively. The number of bound POM molecules to HEWL was 1.04 for the lacunary Keggin POM, and 1.0 for Eu-Keggin, indicating the formation of a 1:1 POM/HEWL complex. The value of 1.38 for Ce-Keggin might indicate a transition from 1:1 to 1:2 interaction.

1. Introduction

Polyoxometalates (POMs) are a variable class of negatively charged inorganic metal-oxygen clusters[1-3]. They consist of oxo-bridged early transition metals in their highest oxidation state. The main advantage of using POMs is their versatility as their chemical and physical properties can be easily altered, resulting in numerous applications. For instance, by changing the size, shape, composition, charge density, solubility, redox potential and acid strength of POMs, diverse applications in the fields of catalysis[4-6], material science[7-9] and medicine[10-13] have been reported. Furthermore, lacunary species, which are formed by the removal of one or more of the addenda atoms from the typical inorganic clusters, can coordinate a broad diversity of different metal ions thereby creating new prospects in POM chemistry. These metal-substituted POMs have a customized coordination chemistry and reactivity[3, 14], and have shown their potential in several catalytic reactions, ranging from the epoxidation of olefins and alkanes to the splitting of water[15-17].

Besides their usefulness in catalysis, numerous POMs have been shown to be biologically active, including anticancer, antiviral, antibiotic, antiprotozoal and antidiabetic activity, both *in vitro* and *in vivo*[10-13]. Nonetheless, the full mechanism behind their medicinal action has not been clarified so far. Several reports have studied the interaction between structurally different POMs and a range of proteins on a molecular level, indicating that the size, shape and charge of the POM as well as the nature of the imbedded metal ion play an important role in these interactions[13, 18-26].

Within our research group, the reactivity of POMs towards several biomolecules and their model systems has been established. Initially, this was illustrated by the hydrolysis of the phospho(di)ester bonds in DNA and RNA model compounds by isopolyoxomolybdates[27-31], isopolyoxovanadates[32, 33] and a series of metal-substituted POMs[34]. Encouraged by

these results the hydrolysis of the highly inert peptide bond in dipeptides, oligopeptides and proteins in the presence of POMs has also been explored. These studies have shown the hydrolytic peptidase activity of oxomolybdate and oxovanadate towards dipeptides and proteins[35-37]. Furthermore, it was found that the peptide bond in a series of unactivated dipeptides and oligopeptides was efficiently hydrolyzed by Zr(IV)- and Hf(IV)-substituted Wells-Dawson[34, 38], Keggin[39] and Lindqvist POMs[40]. In addition, selective hydrolysis of the peptide bond in proteins, for example hen egg white lysozyme (HEWL) and human serum albumin, by a Ce(IV)-substituted Keggin POM and a series of Zr(IV)-substituted POMs, was observed under physiological pH and temperature conditions[20, 41, 42].
The HEWL hydrolysis study in the presence of $[Ce(PW_{11}O_{39})_2]^{10-}$ was the first example of protein hydrolysis by a POM and demonstrates the advantages of POM ligands in the development of artificial peptidases. Firstly, the POM acts as a stabilizing ligand for the hydrolytically active metal ion and secondly, the negatively charged POM is also responsible for the specific interaction with positively charged patches on the protein surface, leading to regioselective hydrolysis. Our previous studies have indicated that the binding is not only directed by an electrostatic interaction between the negatively charged POM structure and positive patches on the surface of HEWL, but also by the coordination of the Ce(IV) ion to the carboxylate side chain of Glu or Asp amino acids[20]. A thorough understanding of the parameters responsible for the interaction on a molecular level is essential in order to tune the selectivity and rationally design metal-substituted POMs with specific interaction properties. In this regard, Eu(III) luminescence studies on the hydrolytically inactive, Eu(III)-substituted Keggin POM will be used to provide information on the molecular interaction that directs the observed selectivity of HEWL hydrolysis. While most techniques studying POM-protein interactions investigate this process from a protein's point of view, Eu(III) luminescence allows to study interaction from a POM's perspective, thereby providing complementary

information. In addition, Eu(III) luminescence spectroscopy allows a direct study of the metal environment during the interaction. Moreover, the concentrations used in luminescence studies resemble those used in hydrolysis studies. This was not the case for most interaction studies except for ITC[43], thus allowing a more precise study of the correlation between binding and hydrolysis. By using Eu(III) luminescence spectroscopy, both the interaction process and the POM speciation can be examined simultaneously as well. Detailed knowledge of the solution speciation of metal-substituted POMs, used for catalytic purposes, is of paramount importance in establishing the active species and further optimization of the catalytic activity. However, it has to be noted that, although lanthanides are characterized by similar chemical and physical properties, the Eu(III) compound used in this study should be considered as a model and not as a structural analogue in the strict sense. Unfortunately, Ce(IV) luminescence spectroscopy is not possible and therefore this Eu(III) compound is the best possible alternative.

Although Eu(III) luminescence spectroscopy has been previously used as a powerful tool for studying the interaction process between various POMs and proteins[19, 26], the link between the molecular interactions and the observed selectivity of metal-substituted Keggin POM catalyzed protein hydrolysis has not been addressed so far. Therefore, in this study the photophysical properties of the hydrolytically inactive $K_4EuPW_{11}O_{39}$ (Eu-Keggin) are examined in the absence and presence of HEWL by steady state and time-resolved luminescence spectroscopy. Tryptophan (Trp) fluorescence spectroscopy will be used to gain information on the interaction from the protein's point of view. In this respect, it is notable that the hydrolysis of HEWL in the presence of $[Ce(PW_{11}O_{39})_2]^{10-}$ (Ce-Keggin) was found to occur at two positions, the Trp28-Val29 (site I) and Asn44-Arg45 (site II) peptide bonds[20]. In addition, the interaction near the Trp28-Val29 site was proposed to be stronger as compared to the second interaction, presumably due to more effective interactions with the

negatively charged POM structure[20]. The intrinsic Trp fluorescence quenching by three Keggin type POMs will be explored. Besides the Eu-Keggin POM, the lacunary Keggin POM ($K_7PW_{11}O_{39}$) and Ce-Keggin will also be used to examine the effect of the substituted metal ion and to determine the strength of interaction using a Stern-Volmer analysis or Tachiya model. Finally, the effect of the electrostatic nature of the interaction will be investigated by studying interactions of Eu-Keggin POM with α-lactalbumin (α-LA), which is structurally highly homologous to HEWL, but has a very different surface potential[44].

2. Materials and Methods

2.1. Materials

Hen egg white lysozyme (HEWL) and α-lactalbumin (α-LA) were purchased from Sigma-Aldrich (St. Louis, Missouri) in the highest available purity and were used without further purification. Potassium chloride, sodium hydrogen carbonate and europium chloride (hexahydrate) were purchased from Acros Organics (Geel, Belgium). Deuterium oxide was obtained from Cambridge Isotope Laboratories Inc. (Andora, Massachusetts). The Eu-Keggin polyoxometalate, $K_4EuPW_{11}O_{39}$, the lacunary Keggin polyoxometalate, $K_7PW_{11}O_{39}$, and the Ce-Keggin polyoxometalate, $[Me_2NH_2]_{10}[Ce(PW_{11}O_{39})_2] \cdot 14\ H_2O$ were synthesized following the reported procedures[45-47] and characterized by ^{31}P NMR spectroscopy[48]. The chemical shifts of $\delta = 5.05$ ppm for the Eu-Keggin and $\delta = -10.6$ ppm for the lacunary Keggin are in accordance with the characteristic values reported in literature[48].

2.2. Luminescence spectroscopy studies

Time-resolved luminescence spectra were recorded on an Edinburgh Instruments FS920 spectrofluorimeter. Quartz cuvettes with 10.0 mm optical path length were used. Spectra were recorded with 5.0 μM Eu-Keggin POM concentration in a phosphate buffer solution

(pH = 7.4) at room temperature because of solubility. Excitation of the sample took place at 291 nm. The emission and excitation slit widths were opened at 0.37 mm (resolution of 1.0 nm). The protein concentration was augmented stepwise from 0 to 5.0 µM. Time-resolved data analysis was performed using Origin Pro 8.0 and the data were fitted as a sum of exponentials of the following form:

$$I(t) = \sum_{i=1}^{n} \alpha_i \exp\left(-t/\tau_i\right) \quad (1)$$

where α_i are the normalised pre-exponential functions and τ_i the lifetime(s).

Steady state luminescence spectra were recorded on an Edinburgh Instruments FS900 steady state spectrofluorimeter. Quartz cuvettes with 10.0 mm optical path length were used. Spectra were recorded with 5.0 µM Eu-Keggin POM concentration in a phosphate buffer solution (pH = 7.4) at room temperature because of solubility, while monitoring the emission at 613 nm. Excitation of the sample took place at 291 nm unless stated otherwise. The emission and excitation slit widths were opened at 0.37 mm (resolution of 1.0 nm). The protein concentration was increased stepwise from 0 to 5.0 µM. The strength of interaction, quantified by the association constant, was calculated from these data with a formula derived from the Stern-Volmer equation[49]:

$$\log \frac{I_{sam} - I_{bl}}{I_{sam}} = \log K_a + n \log [P] \quad (2)$$

where I_{sam} and I_{bl} are the intensity in the presence and absence of the protein, K_a the association constant, n the number of binding sites and [P] the protein concentration.

2.3. Fluorescence spectroscopy studies

Steady state fluorescence experiments were recorded on a Photon Technology Quanta Master QM-6/2005 spectrofluorimeter. Quartz cuvettes with 10.0 mm optical path length were used. Spectra were recorded in a buffered 10 µM protein concentration solution (phosphate buffer, pH = 7.4) at room temperature monitoring the emission from 305 nm to 420 nm, with a maximum at approximately 330 nm. Excitation of the sample took place at 295 nm to avoid excitation of tyrosine residues. The emission and excitation slit widths were opened at 0.37 mm (resolution of 1.0 nm). The Eu-Keggin POM concentration was increased stepwise from 0 to 10 µM with increments of 1.0 µM. The analysis of the results for the Eu-Keggin and lacunary Keggin was done with the help of a derived Stern-Volmer equation[19]:

$$\log \frac{(F_0\text{-}F)}{F} = \log K_q + n \log [Q] \quad (3)$$

where F_0 is the unquenched fluorescence intensity, F the fluorescence in the presence of the quencher and [Q] the concentration of the quencher.

The fluorescence data of the Ce-Keggin was analyzed using a different equation since we expect a number of bound molecules higher than 1. For this reason the Tachiya model, equation (4), is used[50].

$$\log \frac{F_0\text{-}F}{F} = m \log K_q + m \log \left([Q] - [M] \frac{F_0\text{-}F}{F} \right) \quad (4)$$

where F_0 is the unquenched fluorescence intensity, F the fluorescence in the presence of the quencher, [Q] the concentration of the quencher, [M] the concentration of protein and m the number of binding sites.

3. Results and Discussion

3.1. Solution speciation of Eu-Keggin POM

As previously reported[51], metal-substituted Keggin POMs can form different complexes in solution. Factors that influence the solution speciation of these complexes are for instance the

pH and ionic strength of the solution and the concentration of the POM. In our previous work, the characterization of the Eu-Keggin POM in solution was performed at a fixed concentration (100 μM) in the absence of buffer (pH = 6.25)[51]. Under these conditions the formation of a 1:1 Eu-Keggin complex was observed (Fig. 1). However, the concentration of the POM and the presence of buffer may also influence its solution speciation. Moreover, as protein hydrolysis experiments are often performed with varying concentrations of POM in the presence of buffer, it is important to establish the type of species present at different POM starting concentrations. In this study, the effect of the concentration (5.0 μM up to 1.0 mM) of the Eu-Keggin POM on its luminescence properties is investigated at physiological pH by both steady state and time-resolved luminescence spectroscopy. From steady state experiments the concentration effects on the excitation and emission spectrum can be evaluated, while from the time-resolved experiments the Eu(III) speciation can be investigated and therefore, the type of Eu-Keggin POM species in solution can be determined.

Fig. 1.

In a first step, the excitation spectra of the different Eu-Keggin POM solutions were recorded, while monitoring the emission at 613 nm (Fig. 2A). Each of the excitation spectra showed the characteristic Eu(III) excitation lines at 362 nm ($^5D_4 \leftarrow ^7F_0$), 375, 380 and 384 nm (combination of $^5G_2$, $^5L_7$, $^5G_{3\text{-}6} \leftarrow ^7F_{0,1}$), 394 nm ($^5L_6 \leftarrow ^7F_0$), 417 nm ($^5D_3 \leftarrow ^7F_{0,1}$) and 465 nm ($^5D_2 \leftarrow ^7F_0$). In addition, in all of the recorded spectra the presence of a ligand-to-metal-charge transfer (LMCT) band was observed. The maximum of this LMCT band shifted to higher wavelengths with increasing Eu-Keggin concentration: from 255 nm for the 5.0 μM solution to 319 nm for the 1.0 mM solution. Another noticeable feature is the relative intensity of the LMCT band as compared to the most intense Eu(III) transition, namely the $^5L_6 \leftarrow ^7F_0$ transition. For the samples with low concentrations of POM ([Eu-Keggin] < 100 μM) the LMCT band dominates the spectrum, while for higher concentrations (upward of 250 μM) the

$^5L_6 \leftarrow {}^7F_0$ transition becomes the most intense band of the spectrum (Fig. 2B). These effects are probably a result of the faster saturation of the LMCT band as compared to the $^5L_6 \leftarrow {}^7F_0$ transition[52]. This merely means that the deviation of the ideal, linear behaviour concerning photon absorption with increased concentration is larger for the LMCT band.

Fig. 2.

Subsequently, the emission spectra of the Eu-Keggin POM for the used concentrations were measured. The excitation took place at the $^5L_6 \leftarrow {}^7F_0$ transition which is at 394 nm for all the measured concentrations due to the faster saturation of the LMCT band. Four different transitions can be detected in the emission spectra, namely the $^5D_0 \rightarrow {}^7F_0$ (580 nm), $^5D_0 \rightarrow {}^7F_1$ (593 nm), $^5D_0 \rightarrow {}^7F_2$ (613 nm), and $^5D_0 \rightarrow {}^7F_4$ (700 nm) transitions. The emission spectra are depicted in Fig. 3 with the relative increase in intensity for the three main emissions in the inset. From Fig. 3, it can be seen that the intensity of the three main emission transitions increases linearly upon increasing the concentration. The extent of intensity increase over the full concentration range is dependent on the emission band and is lower than expected for all three transitions (92%, 84% and 71% of the expected linear increase for the $^5D_0 \rightarrow {}^7F_1$, $^5D_0 \rightarrow {}^7F_4$ and $^5D_0 \rightarrow {}^7F_2$ transition, respectively).

Fig. 3.

Subsequently, the lifetimes of the excited $^5D_0$ state of the Eu(III) ion were quantified for different Eu-Keggin concentrations. The measurements were performed both in water and deuterated water in order to calculate the number of coordinated water molecules, q(Eu). This can be calculated with the following equation[53, 54]:

$$q(Eu) = A \times [\tau^{-1}_{H_2O} - \tau^{-1}_{D_2O} - C] \quad (5)$$

In Equation (5) $\tau(H_2O)$ and $\tau(D_2O)$ are the measured lifetimes (ms) in water and deuterated water, respectively. The parameters A and C depend on the equation used. For the formula postulated by Beeby *et al.* A is 1.2 ms and C is 0.25 ms^{-1}, while for the formula according to

Supkowski *et al.* A has a value of 1.11 ms and C equals 0.31 ms^{-1}. The number of coordinated water molecules was calculated using both formulas and the obtained values are represented by $q(Eu)_{Be}$ or $q(Eu)_{Su}$, respectively. For every measured concentration a mono-exponential decay was found both in H_2O and in D_2O, indicating the presence of only one type of species at every POM concentration under study. The values of the different lifetimes as well as the number of coordinated water molecules are shown in Table 1 and depicted in Fig. 4.

Table 1.

At low concentrations, there are 4 water molecules coordinated to the Eu(III) ion, indicating the presence of the 1:1 Eu-Keggin POM (Fig. 1). This was also found in previous experiments[51]. Remarkably, q(Eu) decreases linearly when the concentration of Eu-Keggin POM is increased (Fig. 4). The lower value of q(Eu) could indicate the formation of an aggregate at higher concentrations or could be explained by the interaction of the phosphate buffer with the Eu-Keggin POM. Possible aggregation species are the 2:2 Eu(III)-Keggin complex as well as one dimensional polymers[55, 56]. To test this possible aggregate formation, the experiments were performed in the absence of phosphate buffer. In this case, all the lifetimes in H_2O equalled 0.22 ms, while the lifetime in D_2O varied between 1.76 ms up to 1.95 ms. When q(Eu) was calculated according to Equation (5), this led to a coordination number of 4 for all measured samples and thus eliminating the possibility of aggregate formation in the presence of buffer.

Fig. 4.

In subsequent experiments, the effect of the buffer concentration on the luminescence properties of the Eu-Keggin POM was examined. For this purpose, the concentration of Eu-Keggin was kept constant at 1.0 mM, while the phosphate buffer concentration increased from 0 mM up to 10.0 mM in increments of 1.0 mM. The pH remained constant at pH = 7.4. The highest used concentrations of Eu-Keggin POM and phosphate buffer mimic the reaction

conditions in which the hydrolysis of HEWL by a Ce(IV)-substituted Keggin POM was previously observed[20].

From the excitation spectrum, shown in Fig. 5, it can be concluded that there is a clear difference between the sample with and without buffer. This can be seen best when comparing the intensity of the LMCT band (at approximately 310 nm) to the most intense $^5L_6 \leftarrow ^7F_0$ transition. For the sample without phosphate buffer this gives a ratio of 0.36, while for all buffered solutions this ratio drops to a value of 0.14. In addition to quenching, a clear shift of the LMCT band to longer wavelengths (from 310 nm to 319 nm) can be observed. This shift could be explained by an increased bond length between the oxygen atoms of the POM structure and the Eu(III) ion because of the binding of phosphate anions to the Eu(III) ion[57]. This increased bond length results in a more difficult energy transfer from the POM to the Eu(III) ion, which explains the decrease in intensity of the LMCT band.

Fig. 5.

To confirm the substitution of water molecules by phosphate anions in the first coordination sphere of the Eu(III) ion, time-resolved measurements were performed (Table 2). For the unbuffered sample, 4 coordinated water molecules were found, while for the buffer solution with the highest concentration (10.0 mM) only two water molecules were coordinated, indicating that two coordination sites of the Eu(III) ion in Eu-Keggin are replaced. The effect of changes in the buffer concentration on the number of coordinated water molecules is of particular interest for protein hydrolysis which is usually studied using buffered solutions. In peptide bond hydrolysis, a large number of coordinated water molecules is advantageous as these coordinated water molecules are typically replaced by the substrate resulting in activation of the substrate for nucleophilic attack by the remaining water molecules in the first coordination sphere. Therefore, knowledge on the number of coordinated water molecules is paramount to obtain increased reaction kinetics via a close control of the buffer concentration.

Table 2.

The substitution of water molecules by phosphate anions also resulted in a clear increase in the emission intensity (Fig. 6). The relative intensity increase is displayed in the inset of Fig. 6 for the $^5D_0 \rightarrow {}^7F_1$ (593 nm), $^5D_0 \rightarrow {}^7F_2$ (613 nm), and $^5D_0 \rightarrow {}^7F_4$ (700 nm) transitions in the presence of increasing amounts of phosphate buffer. In this graph, the most intense transition, namely the $^5D_0 \rightarrow {}^7F_4$ transition at 700 nm, displayed a more than fourfold intensity increase. The magnetic dipolar transition ($^5D_0 \rightarrow {}^7F_1$) displayed a similar increase with a factor of 4.15 compared to 4.18 for the $^5D_0 \rightarrow {}^7F_4$ transition. The hypersensitive $^5D_0 \rightarrow {}^7F_2$ transition only shows a twofold increase in intensity. Interestingly, this increase occurs in three steps with a plateau between each step. This intensity increase is a result of the gradual substitution of water molecules bound to the Eu(III) ion by phosphate anions (Table 2). For this reason we will use the lowest buffer concentration (1.0 mM) in further experiments, unless stated otherwise.

Fig. 6.

3.2. Eu(III) luminescence of Eu-Keggin POM in the presence of protein

In following experiments, the excitation spectrum of the Eu-Keggin POM was measured upon addition of protein. This was done for three different samples, namely a blank Eu-Keggin solution, a solution containing Eu-Keggin and HEWL in a 10 to 1 ratio and a solution with equal concentrations of Eu-Keggin and HEWL (Fig. 7). When looking at Fig. 7, we can conclude that in the presence of HEWL the characteristic Eu(III) excitation peaks are present and that the number of excitation peaks remains unchanged, however, the position of the LMCT band shifted to higher wavelengths. With increasing concentrations of HEWL, this shift becomes larger and the maximum of the LMCT band shifts from 266 nm for the blank Eu-Keggin solution to 269 nm for the Eu-Keggin/HEWL 10:1 solution, and to 270 nm for the Eu-Keggin/HEWL 1:1 solution. This is the result of an increased bond length between the

oxygen atoms of the POM structure and the Eu(III) ion, presumably as a result of the binding of the POM structure to HEWL[57].

Fig. 7.

Excitation spectra were also recorded in the presence of α-LA. α-LA has a three dimensional structure highly homologous to HEWL, but has a negatively charged surface, while the surface of HEWL is mainly positively charged at physiological pH. Hydrolysis experiments have indicated that the Ce-Keggin is unreactive towards α-LA at physiological conditions, whereas HEWL is cleaved at two positions, as previously noted[20]. Since both proteins have similar 3D structures, this difference in the POM reactivity was attributed to the large variation in the prevailing charges at both protein surfaces[20]. In the spectra shown in Fig. 8, the LMCT band dominates the spectrum, but becomes less dominant when more α-LA is added. The transitions in the spectra also become less intense. It becomes clear that the LMCT band for the samples containing α-LA is comprised of two parts, which have maxima at 257 nm and 301 nm. These two parts are a combination of the LMCT band of the Eu-Keggin and a band coming from the added α-LA. This is evidenced by taking the excitation spectrum of a blank α-LA sample. This spectrum displays a broad band with the maximum intensity at 296 nm. The small shift is a result of the addition of Eu-Keggin to the α-LA solution.

Fig. 8.

In subsequent experiments, the emission spectrum of Eu-Keggin in the presence of HEWL, shown in Fig. 9, was monitored. These spectra display the characteristic Eu(III) emission peaks ($^5D_0 \rightarrow {}^7F_J$ with J = 0-4). The emission bands are located at 580, 593, 613, 651, and 700 nm and correspond to the $^5D_0 \rightarrow {}^7F_0$, $^5D_0 \rightarrow {}^7F_1$, $^5D_0 \rightarrow {}^7F_2$, $^5D_0 \rightarrow {}^7F_3$, and $^5D_0 \rightarrow {}^7F_4$ transitions, respectively. The relative intensity increase for the $^5D_0 \rightarrow {}^7F_1$ (593 nm), $^5D_0 \rightarrow {}^7F_2$ (613 nm) and $^5D_0 \rightarrow {}^7F_4$ (700 nm) transitions in the presence of increasing amounts of HEWL is displayed in the inset of Fig. 9. In this graph, the most intense transition, namely the

hypersensitive $^5D_0 \rightarrow {}^7F_2$ transition at 613 nm, showed more than a threefold intensity increase. The other two transitions show a slightly less pronounced increase, indicating that complex formation leads to lower symmetry around the Eu(III) ion. From the steady state emission spectra the association constant can be calculated by Equation (2). This resulted in a value of 2.2×10^2 M^{-1} for the Eu-Keggin POM/HEWL complex. This value is comparable to previously obtained values of association constants for the Eu-Keggin/HSA and Eu-Keggin/BSA complexes at slightly lower pH values (pH = 7.37 and 6.62, respectively)[51], which were 1.5×10^2 M^{-1} and 2.0×10^3 M^{-1}, respectively. When compared to the reported values for the Eu-Lindqvist POM, lower association constants were obtained for the Eu-Keggin POM[26, 49]. The values were 4.8×10^4 M^{-1} and 4.6×10^3 M^{-1} for the complex with HSA and BSA, respectively. This difference in association constant can be explained by the use of a different protein and by the larger size, different shape and smaller negative charge of the Eu-Keggin as compared to the Eu-Lindqvist POM. In addition, the association constants for Eu-Keggin are calculated with the integrated values of the different transitions, while the association constants for the Eu-Lindqvist POM were calculated by using the maximum transition intensity[49].

Fig. 9.

The local environment of the Eu(III) ion determines the luminescence characteristics of the different transitions. For instance, the transition at 593 nm ($^5D_0 \rightarrow {}^7F_1$) has a magnetic-dipolar origin and therefore should not be strongly affected by the environment of the Eu(III) ion. In contrast, the transition at 613 nm ($^5D_0 \rightarrow {}^7F_2$) is highly affected by the environment because of its electric-dipolar origin. Furthermore, this $^5D_0 \rightarrow {}^7F_2$ transition is hypersensitive to the environment and the intensity of this transition increases with decreasing site symmetry, rendering it to be a good structural probe. Therefore, this transition can be used in combination with the $^5D_0 \rightarrow {}^7F_1$ transition, which serves as a reference, to give an indication of

the changes in the local environment of the Eu(III) ion. The ratio of these two transitions will be abbreviated as I_{613}/I_{593} and the calculated values are given in Table 3. For the blank Eu-Keggin POM a value of 3.17 was found, while the value obtained for a solution containing Eu-Keggin and HEWL in a 1:1 ratio is 4.74. This represents an increase by a factor of 1.49, indicating that the Eu(III) ion is positioned in a different environment with lower symmetry in the presence of HEWL.

Table 3.

When similar experiments were performed with the negatively charged α-LA, the emission spectra (Fig. 10) did not show an increase in intensity as was the case with HEWL. Instead, the intensity decreased by 25% for the Eu-Keggin/α-LA 1:1 solution compared to the blank Eu-Keggin solution. This decrease in intensity, which was also seen in the excitation spectra (Fig. 8), is probably the result of the splitting of the LMCT band in two bands, a combination of the original LMCT band and a newly formed band coming from the added α-LA. Due to the second band, the absorption of photons by the LMCT band and more importantly, the energy transfer from the POM skeleton to the Eu(III) ion becomes less efficient, resulting in a decrease in intensity[58]. If there was interaction between α-LA and Eu-Keggin, the energy could also be back transferred from the protein to the POM. Since this is not the case, these results indicate rather the lack of interaction between Eu-Keggin and α-LA and therefore, explain the lack of reactivity of hydrolytically active analogues of Eu-Keggin towards α-LA, as previously mentioned[20].

Fig. 10.

In a next step, time-resolved luminescence experiments were used to determine the Eu(III) species present in solution in the absence and presence of HEWL. Upon addition of HEWL, it was found that the luminescence decay curves could be fitted with a bi-exponential decay in H_2O and with a tri-exponential decay in D_2O, with a low impact of the third component (0.57-

0.87%). On account of the substitution of water molecules for HEWL, the lifetime of the Eu(III) ion in the resulting Eu-Keggin/HEWL complex increases in water to a value of $\tau(H_2O)$ = 1.22 ms, while the lifetime of the non-bound component (Eu-Keggin POM) remains unchanged at $\tau(H_2O)$ = 0.22 ms. When the HEWL concentration is increased, a decrease in the amount of non-bound Eu-Keggin is observed, while the portion of the Eu-Keggin/HEWL complex displays a concomitant augmentation. This increase is indicative for interaction between the Eu-Keggin POM and HEWL (Fig. 11). In addition, the third component (free Eu(III) ion) disappears when a Eu-Keggin/HEWL ratio of 7:1 is reached, indicating every Eu(III) ion is bound to one Keggin POM. The presence of free Eu(III) ion is probably a result of the partial dissociation of the Eu-Keggin in phosphate buffer[59, 60].

The experiments were repeated in D_2O to calculate the number of water molecules coordinated to the Eu(III) ion in Eu-Keggin. The average lifetime of the second component in D_2O ($<\tau_2>$) amounts to 1.15 ms. Based on Equation (5), the calculation of the hydration number, q(Eu), of the formed Eu-Keggin/HEWL complex resulted in a negative value, q(Eu) = -0.39, indicating the formation of a non-hydrated species because of the substitution of all 4 coordinated water molecules by HEWL. The intensity increase, which was seen in Fig. 9, is a result of this substitution of water molecules bound to the Eu(III) ion by HEWL amino acid residues. The luminescence of the Eu(III) ion is highly quenched by bound water molecules due to the deactivation of the $^5D_0$ excited state by coupling with OH oscillators. When water molecules are substituted for HEWL, this deactivation and hence, the quenching of the Eu(III) luminescence is decreased. As a consequence, higher intensities of the Eu(III) luminescence are observed.

Fig. 11.

With the negatively charged α-LA the time-resolved experiments yielded very different results. When α-LA was added the decay curves could be fitted with a mono-exponential decay, τ = 0.22 ms, indicating there is no formation of a Eu-Keggin/α-LA complex. These data indicate again the absence of interaction between α-LA and Eu-Keggin. In addition, the POM speciation remains the same, as only the 1:1 Eu-Keggin species was detected in solution for all the measured ratios.

3.3. Tryptophan Fluorescence

HEWL is a 14.6 kDa single chain protein and consists of 129 amino acid residues including 6 Trp residues, 3 tyrosine (Tyr) residues and 4 disulfide bonds. Emission of HEWL is dominated by the Trp residues, which absorb at the longest wavelength and display the largest extinction co-efficient. Moreover, energy absorbed by phenylalanine (Phe) and Tyr residues is often efficiently transferred to the Trp residues in the same protein[61]. In these steady state fluorescence experiments, the protein concentration was kept constant (10 μM), while the POM concentration was increased stepwise from 0 to 10 μM with increments of 1.0 μM. The quenching of the HEWL fluorescence in the presence of the Eu-Keggin is shown in Fig. 12. The results were analyzed using a derived Stern-Volmer equation, (Equation (3)) and in the inset of Fig. 12, the derived Stern-Volmer plot for the quenching of HEWL fluorescence by Eu-Keggin is given. To account for the charge of the POM, these measurements are also done for the lacunary Keggin POM, which contains 7 negative charges compared to 4 for the Eu-Keggin. In addition, it is possible to investigate the effect of the hydrolytically active Ce-Keggin POM on Trp fluorescence thereby providing direct evidence for the interaction between this POM and HEWL. However, since multiple binding sites are expected, analysis of these results has to be carried out by the Tachiya model (Equation (4)). The main benefit of using these equations is the opportunity of extracting the quenching constant (K_q) and the

number of bound molecules (n or m). The calculated values of the quenching constants are given in Table 4.

Table 4.

Formation of a 1:1 complex was observed for both the lacunary Keggin ($K_7PW_{11}O_{39}$) and Eu-Keggin, which is representative for a specific binding between these Keggin type POMs and the HEWL surface at the highly positively charged site I. Although integer values of n are expected, the value of 1.38 for Ce-Keggin is indicative for a transition from a 1:1 to a 1:2 binding, as was previously reported[43]. This is the result of the combination of the strong, POM directed interaction at site I (Trp28-Val29) and the weaker, metal-directed interaction at the second cleavage site of HEWL (Asn44-Arg45). This strong interaction near the Trp28-Val29 peptide bond in the α-helical part of the structure was already evidenced by ^{15}N, ^{1}H HSQC NMR at room temperature, whereas interaction near the second hydrolytic position in the β-sheet region was less explicit from these experiments[20]. However, in more recent thermodynamic studies, the Ce-Keggin POM indeed showed a 1:1 interaction with HEWL at 25 °C. In addition, it should be mentioned that no hydrolysis occurred during these interaction experiments since the measurements were performed at room temperature. Hydrolysis of HEWL by the Ce-Keggin POM was only observed upon increasing the temperature to 37 °C. However, a second interaction, presumably near the Asn44-Arg45 peptide bond, was observed when increasing the temperature to 37 °C at which these hydrolysis experiments were performed[43]. _ENREF_20In addition, there are no Trp residues in the vicinity of the second cleavage site (Asn44-Arg45), with the closest Trp residues being more than 10 Å away, whereas the first site contains a Trp residue at the upstream position (residue 28).

The small difference in the quenching constant between the lacunary Keggin and Eu-Keggin POM can be attributed to the electrostatic nature of the interaction. The lacunary Keggin has 7 negative charges. In contrast, the Eu-Keggin bears only 4 negative charges, which results in a

larger quenching constant for the lacunary Keggin POM. The results obtained in this work, are comparable to the results that were previously reported for the Eu-Keggin/HSA complex[51]. However, a larger quenching constant was obtained upon addition of the Ce-Keggin. The larger quenching constant can be explained by an increased POM/HEWL ratio. This can be seen by the increased number of bound molecules per HEWL, indicating the transition from a 1:1 to a 1:2 binding[43].

When fluorescence quenching experiments were carried out with the Eu-Keggin POM and α-LA, a quenching constant of 5.5×10^{2} M^{-1} was found. This value is approximately 20 times lower as compared to the value obtained with HEWL. In addition, the number of bound Eu-Keggin POMs per α-LA was 0.64. Both these results indicate that there is a very weak interaction between Eu-Keggin and α-LA. This interaction is probably driven by the positively charged Eu(III) ion and the negatively charged surface of α-LA, but is too weak to be determined by luminescence spectroscopy. This again is in accordance with previous hydrolysis experiments, which did not result in observable cleavage of α-LA in the presence of the Ce-Keggin at physiological pH[20]. More recent thermodynamic experiments in addition did not result in an observable interaction between the Ce-Keggin and α-LA at a pH value of 7.4, either due to a lack of interaction or due to an affinity constant lower than the observable limit for K_a values in calorimetric measurements.

Fig. 12.

4. Conclusions

In this article we account for further steps towards the comprehension of the parameters responsible for the interaction between hydrolytically active polyoxometalate analogues and proteins on a molecular level. As our preliminary results have shown that Ce(IV)-Keggin also efficiently hydrolyzes proteins other than HEWL, the further optimization of the hydrolytic

selectivity of metal-substituted POMs as artificial proteases depends on these thermodynamic parameters.

The Eu(III)-substituted Keggin POM shows increased emission intensity upon addition of HEWL. Upon addition of α-LA, a protein which is structurally highly homologous to HEWL, but has a largely negative surface potential, no intensity increase was observed. These results are in accordance with both the previously reported hydrolytic reactivity of a Ce(IV)-substituted Keggin type POM towards HEWL and its lack of reactivity towards the α-LA substrate. Fluorescence experiments performed with the lacunary and Eu(III)-substituted Keggin POM, showed the formation of a 1:1 POM:HEWL complex which is in accordance with previous results and presumably can be attributed to binding of the POM near the Trp28-Val29 cleavage site. These experiments were also carried out with Ce-Keggin. Ce-Keggin showed the strongest interaction as a result of the increased Lewis acidity of Ce(IV) as compared to Eu(III). The number of bound molecules (1.38) for Ce-Keggin might indicate a transition from a 1:1 to a 1:2 interaction.

The interaction studies presented in this paper complement ongoing research in our group which focuses on the hydrolysis of proteins in the presence of different POMs and contribute towards the establishment of a structure-activity relationship of metal-substituted polyoxotungstates as a new class of metalloproteases and hence, to the faster development of these polyoxotungstates as artificial peptidases.

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Table 1. Time-resolved decay data for the Eu-Keggin POM at different concentrations (pH = 7.4, phosphate buffer to Eu-Keggin ratio 10:1). Excitation of the Eu-Keggin POM took place at 394 nm. The emission was monitored at 613 nm.

Concentration Eu-Keggin (μM)	**5.0**	**10**	**50**	**100**	**250**	**500**	**1000**
$\tau(H_2O)$ (±0.01 ms)	0.22	0.24	0.24	0.24	0.25	0.29	0.41
$\tau(D_2O)$ (±0.01 ms)	2.23	2.88	3.06	2.88	2.60	2.37	2.93
$q(Eu)_{Be}$ (± 0.5)	4.50	4.37	4.27	4.21	3.99	3.33	2.21
$q(Eu)_{Su}$ (± 0.5)	4.09	3.98	3.88	3.83	3.63	3.02	1.97

Table 2. Time-resolved decay data for Eu-Keggin POM (1.0 mM) with different phosphate buffer concentrations (pH = 7.4). Excitation of the Eu-Keggin POM took place at 394 nm. The emission was monitored at 613 nm.

[Buffer] (mM)	**0**	**1.0**	**2.0**	**3.0**	**4.0**	**5.0**	**6.0**	**7.0**	**8.0**	**9.0**	**10.0**
$\tau(H_2O)$ (±0.01 ms)	0.23	0.27	0.27	0.28	0.29	0.30	0.29	0.31	0.28	0.35	0.41
$\tau(D_2O)$ (±0.01 ms)	2.44	3.18	2.87	2.77	2.58	2.64	2.55	2.48	2.49	2.43	2.48
$q(Eu)_{Be}$ (± 0.5)	4.44	3.69	3.75	3.49	3.38	3.28	3.32	3.03	3.45	2.67	2.14
$q(Eu)_{Su}$ (± 0.5)	4.04	3.35	3.41	3.17	3.06	2.97	3.00	2.74	3.13	2.40	1.92

Table 3. Ratio between the $^5D_0 \rightarrow {}^7F_2$ (613 nm) and $^5D_0 \rightarrow {}^7F_1$ (593 nm) transitions for the Eu-Keggin POM (5 µM) in the presence of increasing amounts of HEWL. The concentration of Eu-Keggin POM was kept constant at 5 µM (pH = 7.4).

Ratio POM/Protein	**Blank**	**10:1**	**8:1**	**7:1**	**5:1**	**2:1**	**1:1**
I_{613}/I_{593} Eu-Keggin/Lys	3.17	3.47	4.23	4.34	4.55	4.61	4.74

Table 4. Calculated values of the quenching constants for different POM-HEWL complexes at pH = 7.4.

POM	K_q (M^{-1})	Bound Molecules
$K_7PW_{11}O_{39}$	9.1 (± 0.2)×10^4	1.04 (± 0.01)
$K_4EuPW_{11}O_{39}$	4 (± 1)×10^4	1.0 (± 0.1)
$[Me_2NH_2]_{10}[Ce(PW_{11}O_{39})_2]$	4.1 (± 0.4)×10^5	1.38 (± 0.02)

Figure Captions

Fig. 1. Combined polyhedral/ball-and-stick representations of the different possible Eu-Keggin POMs. The 1:1 monomer is shown in (A) and the 1:2 dimer in (B). Green octahedra: WO_6, red tetrahedra: PO_4, blue: Eu(III) ion, black: oxygen atoms of coordinated water molecules.

Fig. 2. Excitation spectra of Eu-Keggin POM solutions with different concentrations while monitoring the emission at 613 nm (A) and with normalized intensity (B). The used concentrations were 5.0 (black), 10 (red), 50 (blue), 100 (dark cyan), 250 (magenta), 500 (dark yellow) and 1000 µM (navy), at pH = 7.4. The phosphate buffer was used in a tenfold excess.

Fig. 3. Steady state emission spectra of Eu-Keggin POM solutions with different concentrations ranging from 5.0 µM to 1000 µM with excitation at 394 nm. The pH of the solution was 7.4 and the phosphate buffer was used in a tenfold excess. In the inset the relative intensity increase is depicted for the 3 main emission transitions: $^5D_0 \rightarrow {}^7F_1$ (593 nm, black squares), $^5D_0 \rightarrow {}^7F_2$ (613 nm, red dots) and $^5D_0 \rightarrow {}^7F_4$ (700 nm, blue triangles).

Fig. 4. Number of coordinated water molecules, q(Eu), in function of Eu-Keggin concentration.

Fig. 5. Normalized excitation spectra of Eu-Keggin POM solutions with different phosphate buffer concentrations (pH = 7.4) while monitoring the emission at 613 nm. The concentration of Eu-Keggin was kept constant at 1.0 mM while the concentration of buffer increased stepwise (1.0 mM) from 0 mM up to 10.0 mM.

Fig. 6. Steady state emission spectra for the Eu-Keggin POM (1.0 mM) upon increasing concentration of phosphate buffer (0-10.0 mM, pH = 7.4) in aqueous solution. Excitation of the samples took place at 394 nm. In the inset the relative intensity increase is depicted for the 3 main emission transitions: $^5D_0 \rightarrow {}^7F_1$ (593 nm, black squares), $^5D_0 \rightarrow {}^7F_2$ (613 nm, red dots) and $^5D_0 \rightarrow {}^7F_4$ (700 nm, blue triangles).

Fig. 7. Excitation spectra of Eu-Keggin POM in the absence and presence of HEWL. Black solid curve: blank Eu-Keggin solution; red dashed curve: 10:1 Eu-Keggin POM/HEWL and blue dotted curve: 1:1 Eu-Keggin POM/HEWL. The POM concentration was kept constant at 5 µM due to solubility issues in a phosphate buffer solution (100 µM, pH = 7.4). The emission was monitored at 613 nm.

Fig. 8. Excitation spectra (A) and normalized excitation spectra (B) of Eu-Keggin POM in the absence and presence of α-LA. Black solid curve: blank Eu-Keggin solution; red dashed curve: 10:1 Eu-Keggin POM/α-LA; blue dotted curve: 1:1 Eu-Keggin POM/α-LA and dark cyan dot-dashed curve: blank α-LA solution (100 μM). The POM concentration was kept constant at 100 μM in a phosphate buffer solution (1000 μM, pH = 7.4). The emission was monitored at 613 nm.

Fig. 9. Steady state emission spectra for the Eu-Keggin POM (5 μM) upon increasing concentration of HEWL (0-5 μM) in aqueous solution. The phosphate buffer concentration was 100 μM at pH = 7.4 and excitation took place at 291 nm. In the inset the relative intensity increase is depicted for the 3 main emission transitions: $^5D_0 \rightarrow {}^7F_1$ (593 nm, black squares), $^5D_0 \rightarrow {}^7F_2$ (613 nm, red dots) and $^5D_0 \rightarrow {}^7F_4$ (700 nm, blue triangles).

Fig. 10. Emission spectra of Eu-Keggin POM in the absence and presence of α-LA. Black solid curve: blank Eu-Keggin solution; red dashed curve: 10:1 Eu-Keggin POM/α-LA and blue dotted curve: 1:1 Eu-Keggin POM/α-LA. The POM concentration was kept constant at 100 μM in a phosphate buffer solution (1000 μM, pH = 7.4). The excitation took place at 291 nm.

Fig. 11. Change of the species present in solution (in %) upon addition of HEWL (0-10 μM) to a solution of Eu-Keggin POM (5 μM, in phosphate buffer: 100 μM, pH = 7.4). The shorter-lived component (0.22 ms) corresponds to the lifetime of the free Eu-Keggin POM and was fixed in the measurement, while the longer-lived component (1.22 ms) corresponds to the lifetime of the formed POM/HEWL complex.

Fig. 12. Emission fluorescence spectra of HEWL in the absence and presence of different concentrations of Eu-Keggin POM ([HEWL] = 10 μM, phosphate buffer concentration 100 μM at pH = 7.4). From top to bottom, the concentration of Eu-Keggin POM increased stepwise from 0 to 10 μM with increments of 1.0 μM. The excitation wavelength was 295 nm. In the inset, the plot of the derived Stern-Volmer equation is given (with $R^2 = 0.99$). From the plot, K_q and n were calculated to be 4×10^4 M^{-1} and 1.0, respectively.